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FOREWORD

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CONCLUSIONS

7q contains at least two tumor suppressor genes that can be inactivated in breast cancers, one at 7q22 and one at 7q31

LOH mapping analysis and protein-protein interaction studies, strongly point towards the CUTL1 gene as a candidate tumor suppressor gene

LOH of CUTL1 would likely not be a useful prognostic marker for breast tumors

REFERENCES

INTRODUCTION

Background

Intensive research in the past 20 years has established that cancer is caused by multiple genetic changes that affect two classes of genes, the proto-oncogenes and tumor suppressor genes. In human breast cancer, genetic deletions have been demonstrated to be one of the major genetic abnormalities. LOH has been documented at several chromosomal locations, including 1p, 1q, 2p, 3p, 6q, 7q, 8q, 9q, 11p, 11q, 13q, 15q, 16q, 17p, 17q, 18p, 18q, and 22q (1-5) {Reviewed in (6)}. So far only a few of these regions of LOH have been shown to include a known tumor suppressor gene implicated in breast cancer, such as *p53* at chromosome 17p13 (7-9), *BRCA-1* at 17q21 (10) and *BRCA-2* at 13q12-13 (11).

We have recently identified a transcription factor, the human Cut (hCut) transcriptional repressor, that represses expression of c-Myc, a proto-oncogene that was found to be activated in several cancers including breast cancers (12). Amplification of c-Myc has been observed in 4% to 40% of breast carcinomas (13-28)[reviewed in (29)]. In one study of 282 patients with a median follow-up of 74 months, c-Myc amplification was of greater prognostic significance than were *erbB2* and hormone status and it was second to nodal status in multivariate analysis (15). However, tumors with normal gene copy number still showed a low ratio (43%) of disease-free survival after 5 years (15). One possible explanation for this is that, as seen in other cancers, gene amplification represents only one of the various mechanisms leading to c-Myc overexpression in breast tumors. In my grant proposal, I predicted that in a subset of breast tumors, c-Myc would be activated as a result of an alteration in one of its mechanisms of regulation.

The CUTL1 (Cut-like 1) gene was mapped to chromosome 7, band 7q22, a chromosomal region that is frequently rearranged in some human cancers. Two sets of data suggested to us that CUTL1 may be altered in some breast cancers.

First, female transgenic mice expressing the Polyomavirus (PyV) Large T (LT) antigen under the control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) frequently develop, in addition to mammary tumors, uterine leiomyomas (30). Since we had identified LOH at CUTL1 in human uterine leiomyomas, we examined whether PyV LT antigen formed specific complexes not only with members of the retinoblastoma (Rb) family (p105Rb, p107, p130), but also with the mammalian Cut protein. The results of coimmunoprecipitation analyses revealed that specific complexes of Cut and PyV LT antigen could be detected in both leiomyomas and mammary tumors. Since viral oncoproteins effect cellular transformation by interacting with and modulating the action of cellular proteins, the existence of LT/Cut complexes suggested that alterations in the function of the Cut protein may be an important event in the etiology of breast cancer. These results have now been published (30).

The above results lead us to hypothesize that genetic alterations within the 7q22 region may also occur in human breast tumors. In a pilot study, we identified LOH at CUTL1 in 6 of 63 informative patients (9.5%). Using polymorphic markers covering the entirety of 7q, we found that the region of LOH was very large and included 7q31 as well as 7q22 in 4 of these 6 cases, while in the 2 remaining cases only 7q22 was deleted. These results suggested that two regions of the long arm of chromosome are deleted in a fraction of breast cancers: 7q22 and 7q31.

BODY

Hypothesis

We hypothesized that the human Cut gene, CUTL1, functions as a tumor suppressor gene whose deletion and/or mutation represents an important event in the etiology of some breast tumors.

Specific Aims

Our **specific aims** were to use polymorphic markers within and around CUTL1 to determine whether CUTL1 is situated in the minimal region of 7q22 that is deleted in some breast tumors, to verify whether CUTL1 is mutated in these tumors and to determine whether LOH of CUTL1 can be used as a prognostic marker for breast tumors.

Summary of Progress

During this last year, we have made progress on several fronts:

- we determined the complete CUTL1 exon/intron structure, including the sequence at the 5' and 3' end of introns.
- we identified novel alternatively spliced products of the CUTL1 gene.
- we have mapped three polymorphic markers within the boundaries of the CUTL1 gene.
- we determined the frequency of LOH of CUTL1 in patients with sporadic breast cancer using tumor samples from the Royal Victoria Hospital, the Manitoba Breast Tumor Bank and Dr. Rosette Lidereau.
- using samples from the Manitoba Breast Tumor Bank, we verified whether any correlation existed between LOH of CUTL1 and other clinical or pathological parameters.

I will detail below the results we have obtained in each of these areas.

CUTL1 Exon/Intron Structure

One of our long term goals is to determine, in breast tumors with CUTL1 LOH, whether the remaining allele is mutated. For most tumors it will not be possible to obtain RNA that would serve to clone CUTL1 cDNA. Thus, the search for mutation will be performed with genomic DNA. For this purpose, it is necessary first to determine the CUTL1 exon/intron structure, including the sequence at the 5' and 3' end of introns. The sequence information obtained in this manner will then serve to design oligonucleotides that can be used as primers to PCR-amplify individual exons as well as some of the flanking intronic sequences.

We have now determined the complete exon/intron structure of the CUTL1 gene (Fig. 1, page 4). Starting from a panel of cosmid clones encompassing the entirety of the gene, we performed DNA sequencing using as primers oligonucleotides derived from the cDNA sequence. In addition, DNA sequencing of the long arm of chromosome 7, as part of the human genome project, has generated a lot of sequence information (<http://www.ncbi.nlm.nih.gov>, accession number AF024533 & AF047825). The chromosome 7 DNA sequence was analyzed periodically to identify stretches that would contain CUTL1 cDNA sequence. In this manner, we have confirmed some of the results obtained through our own DNA sequencing project and found some exon/intron junctions not yet identified. DNA sequence at the exon/intron junction is presented in Table 1, page 5. Also shown in Fig. 1 are alternatively initiated or spliced products of the CUTL1 gene that we have identified. The biochemical activities of their encoded proteins are currently being investigated.

A genomic DNA fragment corresponding to the 5' end of the CUTL1 cDNA was subcloned into a pKS vector. No TATA box was found within approximately 1 Kbp of DNA sequence. A riboprobe was made and used in RNase mapping analysis to map the transcription start site(s) of the gene (Fig. 2, page 6). Several protected fragments were observed, suggesting that transcription starts at various positions, a finding that is consistent with the absence of a TATA box at the 5' end of the gene.

The data accumulated on the CUTL1 exon/intron structure, transcription start sites and alternatively spliced products form the basis of a manuscript that will be submitted soon .

Mapping of Polymorphic Markers Relative to CUTL1

We had previously identified three polymorphic markers within or close to the CUTL1 gene: D7S515, D7S666 and D7S518 (31). The cloning of CUTL1 genomic DNA allowed us to precisely map these markers by Southern blot analysis. The positions of these three markers are shown in Fig. 1, page 4. D7S515 is located within intron 3, D7S666 in intron 6 and D7S518 in intron 20. Thus, all three markers are positioned within the CUTL1 gene.

Table 1 Nucleotide Sequence of Exon/Intron Junctions of the CUTL1 Gene

CUTL1 exon #	5' end	3' end	5'end sequence of exon	3'end sequence of exon	Intron size (bp)
1	1	73		CAGGTTGAAGgtgagcggcgt	>9300
2	74	184	ttccccaacagAGAGAACTCG	CACTCCAGAGgtgagggcgcgt	>44600
3	185	232	ctcctgctccagGATTTGCGCA	CCAAGGAGAGgtaagctttt	41309
4	233	311	ttccttccctttcagATTGATGCAC	GACGTCCCAGgtaagcccc	27029
5	312	449	tttcctgttggtgcagATCCGTACC	AAAAATCAAGgttggtggaaa	6707
6	450	573	ttttcttttctgcagAGGTTACGAT	AAAAGGAGAGgtgagcatgactt	7371
7	574	650	tcttcctttgcagAAAGCTGCAG	CTACAAACAGgttttgatactctcc	>23500
8	651	717	tgcctttcagCCCTGGAAAA	CTACTGCAAAgtaagtctc	>18600
9	718	766	caccctcctagGGCCGACGAG	GGCAAACCAGgtaggaccctgg.	11610
10	767	871	ttccttcagAGGGCAGAGG	ACCAGACGTGgtgggtagccc	7971
11	872	1060	ttctctccccagGAGCAGGCCA	CACACTCAAAgtaagggggctg	19263
12	1061	1119	ttctctgttttcagCAACTGGAAG	AAGAGCTGAAgtaagtacggagag	3969
13	1120	1168	gttggtgctcttgagCATCTGAAG	TGGGACACAGgtacgtgtctcacctc	1614
14	1169	1265	ctctgcccccttctagGATGCGGCCA	GACCTGAGCGgtaggttggccgggct	1051
15	1266	1937	tgcccttccttgcagGGTCAGCCAG	AGACAAAGAGgtgagagactggcgt	1495
16	1938	2003	cttggttttcttctagAGAATCCAGG	GGGTCTGAAGgtatgttgaggcagg	1202
17	2004	2105	aacttctccccacagGTAACATCAC	CAGAAACTGgtacagcttccattt	1186
18	2106	2950	tgtttctccatgcagCAGAGCCGGC	CGGGGAGAAGgtaagggatctgctct	2185
19	2951	3116	cggtgccactccagGTGCTGGGCC	CAAGGGCCAGgtaatgggggtcctgc	556
20	3117	3173	ctttttctacttttagTCCTCCACTC	GTGAGCTCAGgtaagcagcagtttc	22184
21	3174	3476	gtgctttaattacagAAAGCACTCC	AACAACCTCGgtaggttctcctc	6374
22	3477	3665	ctgttttctctctagGCCAGCGCTT	GAGAAGAAAGgtaagtctccctgccc	5077
23	3666	3930	tctcttcctccgcagCTACATGAA	ACAACCTACAGgtacgacggctggcac	8826
24	3931	5375	cttggtgtcttgcagGTCTCGGATC	TTACTCCACAtatTTTTTaaacaaaaa	

Nucleotide numbers at the 5' and 3' ends of exons corresponds to that of the CUTL1 cDNA sequence (GenBank, accession No. M74099). Intron and exon nucleotide sequences are shown in small and capital letters, respectively.

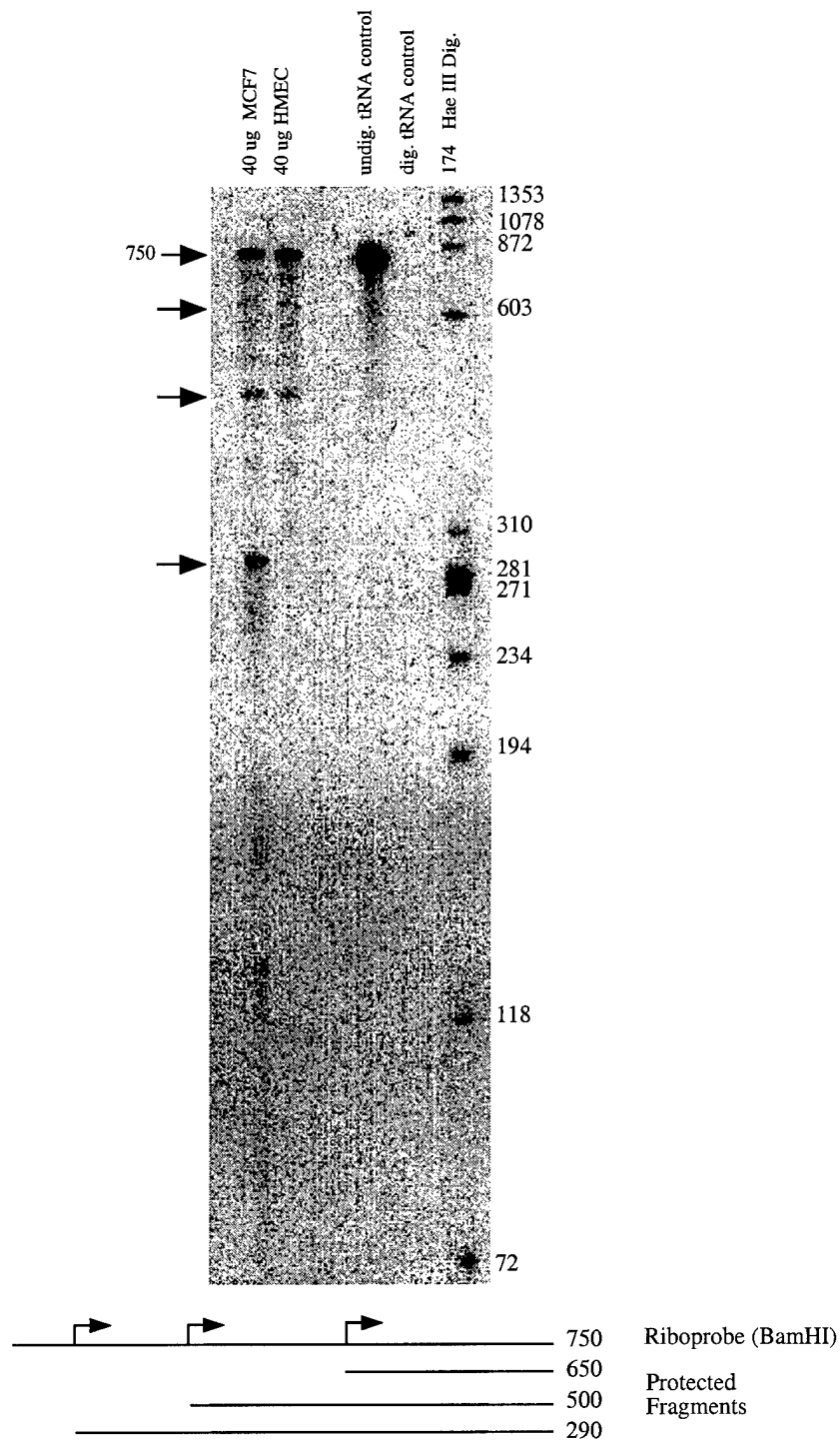


Fig. 4 RNase Mapping Analysis of CUTL1 mRNA 5' Ends

A genomic fragment corresponding to the 5' end of the CUTL1 cDNA was subcloned into the pKS Bluescribe vector (Stratagene). A riboprobe was made and used in RNase mapping analysis using total RNA from the MCF7 breast tumor cell line and human mammary epithelial cells (HMEC). The undigested riboprobe as well as protected fragments are indicated by arrows.

LOH of CUTL1 in Breast Tumors

As a first step to verify whether LOH of 7q22 may occur in breast cancer, we analyzed breast tumor DNA samples that had previously been characterized regarding LOH of 7q31, the chromosomal region adjacent to 7q22. We and others had shown that within 7q22 the marker D7S518 is the most frequently deleted in uterine leiomyomas (31, 32). Thus, we first asked whether breast tumors with or without LOH of 7q31 could exhibit LOH of D7S518. This marker has been mapped to intron 20 of the CUTL1 gene ((31) and Soucie et al. , manuscript in preparation).

A total of 33 pairs of samples from the Bièche et al. study were analyzed (33). LOH of D7S518 was found in 5 of 10 cases with LOH of 7q31 and in 1 out of 23 cases without LOH of 7q31 (Table 2).

A total of 63 pairs of samples from the Lin et al. study were analyzed (34). Among 9 pairs of samples with LOH of 7q31, 7 were informative for D7S518 and 4 exhibited LOH of this marker (Table 2). Among 54 pairs of samples without LOH of 7q31, 2 had suffered LOH of D7S518. Results obtained with these two sets of samples demonstrated that among breast tumors with LOH of the 7q31 region, 53% (9/17) also exhibit LOH of the D7S518 marker in 7q22. It is likely that the deleted region in these tumors is large and encompasses both 7q22 and 7q31. On the other hand, among breast tumors without LOH of 7q31, a small fraction suffered LOH of D7S518. Thus, LOH of D7S518 can be observed independently of LOH at 7q31. We conclude that the long arm of chromosome 7 contains at least two tumor suppressor genes that can be inactivated in breast cancers, one at 7q22 and one at 7q31.

Table 2 Loss of Heterozygosity of D7S518 in Breast Tumors With or Without LOH of 7q31

Source of Samples	7q31 Status in Breast Cancers	Samples Analyzed	Informative Cases for D7S518	LOH of D7S518
Bièche et al. Study	LOH of 7q31	10	10	5 (50%)
	Retention of 7q31	23	23	1 (4%)
Lin et al. Study	LOH of 7q31	9	7	4 (57%)
	Retention of 7q31	54	54	2 (3.7%)

DNAs from breast cancers and matched normal tissues were amplified from patients with breast cancers using oligonucleotide primers for the polymorphic marker D7S518. The source of samples is indicated, together with the numbers of patients tested, informative patients and patients with LOH for markers on 7q31 or 7q22.

To establish the incidence and extent of 7q22 deletion, we investigated LOH of 7q22 in samples from the National Cancer Institute of Canada (NCIC)-Manitoba Breast Tumor Bank (35). We have analyzed 66 pairs of tumors and adjacent normal control tissues representative of all classes of breast tumors. At least three microsatellite markers within 7q22, *D7S518*, *D7S515* and *D7S666*, were used in all cases. In a previous study, we have mapped the marker *D7S518* within an intron of the CUTL1 gene and the markers *D7S515* and *D7S666* close to the 5' end of this gene (31). Determination of the CUTL1 exon/intron structure and DNA sequencing of the long arm of chromosome 7 has since revealed that all three markers are located within the CUTL1 gene, in introns 20, 3, and 6 respectively (Fig. 1, page 4) (Soucie et al., manuscript in preparation; <http://www.ncbi.nlm.nih.gov>, accession number AF024533 & AF047825). Tumors with LOH of one or more of these markers were then further analyzed to map the boundaries of the deleted regions. The physical order of all of the microsatellite markers and CUTL1 with respect to each

other have previously been established (31). The number of cases studied for each marker, the number of informative cases, and the number and percentage of cases that exhibit LOH are given in Table 3 and shown in Fig. 4, page 10. Representative LOH results are shown in Fig. 3, page 9. LOH was scored with at least one marker in 12 of 66 (18.2%) of the tumors examined (patients 10544, 11305, 93-4635, 93-5199, 93-11232, 93-11747, 93-12017, 93-18747, 94-1663, 94-3808, 94-5629, and 94-133582, see Fig. 4, page 10). The superposition of the overlapping deletions in the 12 tumors revealed two common regions of deletion, one in 7q22 and encompassing the CUTL1 gene, and one in 7q31 (Fig. 4). The proximal (centromeric) and distal (telomeric) boundaries of the critical region in 7q22 were defined by breakpoints in tumors #10544, 11305, and #93-5199 that were flanked by the *D7S666* and *D7S658* markers, respectively (Fig. 4). The proximal (centromeric) and distal (telomeric) boundaries of the critical region in 7q31 were flanked by the *D7S480* and *D7S650* markers, respectively (Fig. 4).

Table 3**LOH Analysis of 66 Breast Cancers Using Polymorphic Markers on Chromosome 7.**

Markers	Patients tested	Informative patients	Patients with LOH
D7S524	12	5	2
D7S527	3	2	0
D7S518	66	59	6
D7S666	66	47	4
D7S515	66	48	8
D7S658	5	3	0
D7S471	1	1	0
D7S486	9	8	3
D7S522	12	9	3
D7S480	12	12	3
D7S650	12	10	3

DNAs from tumors and matched normal peripheral breast tissues were amplified from 66 patients with breast cancers using oligonucleotide primers for 11 polymorphic markers on chromosome 7q. The list of markers is presented, together with the numbers of patients tested, informative patients and patients with LOH for each marker. The level of informativeness observed for each of the markers in our cohort of patients was consistent with the published values.

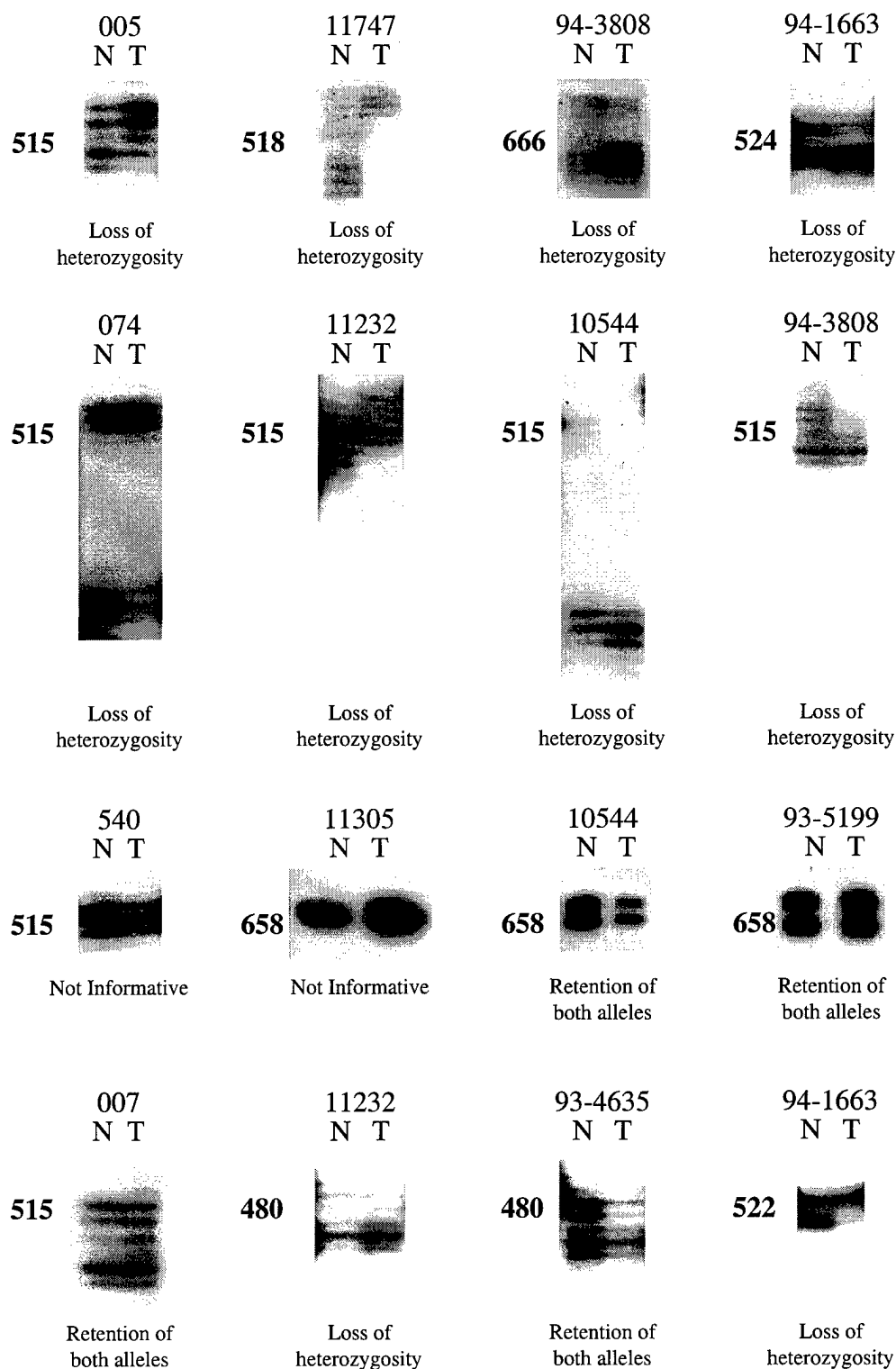
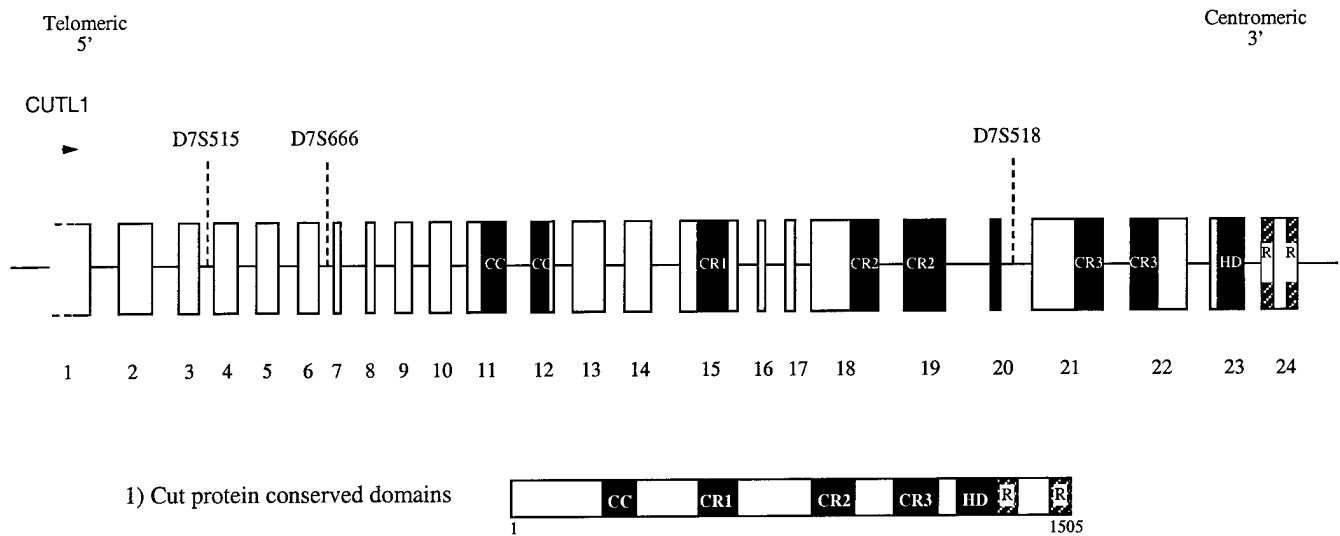


Fig. 3 Representative PCR amplifications of (CA)_n microsatellite repeats

Representative PCR amplifications of (CA)_n microsatellite repeats D7S518, D7S666, D7S524, D7S515, D7S522, D7S480, D7S650, D7S658 are shown. Oligonucleotide primers were used to PCR amplify the regions of DNA containing these markers, in the presence of radiolabeled dCTP. Products were denatured and separated on a standard 5% sequencing gel. DNAs from tumors (T) and matched normal breast tissues (N) were amplified from 66 patients with breast cancer. A patient is considered to be informative if there are 2 major bands (corresponding to 2 alleles) in the normal DNA lane. A patient shows LOH if, in the tumor DNA lane, one of the alleles is absent or shows diminished intensity. For example, patient 10544 is informative for the marker D7S515, and shows LOH at that locus, but shows no LOH at locus D7S658, and patient 11305 is uninformative for marker D7S658.



2B

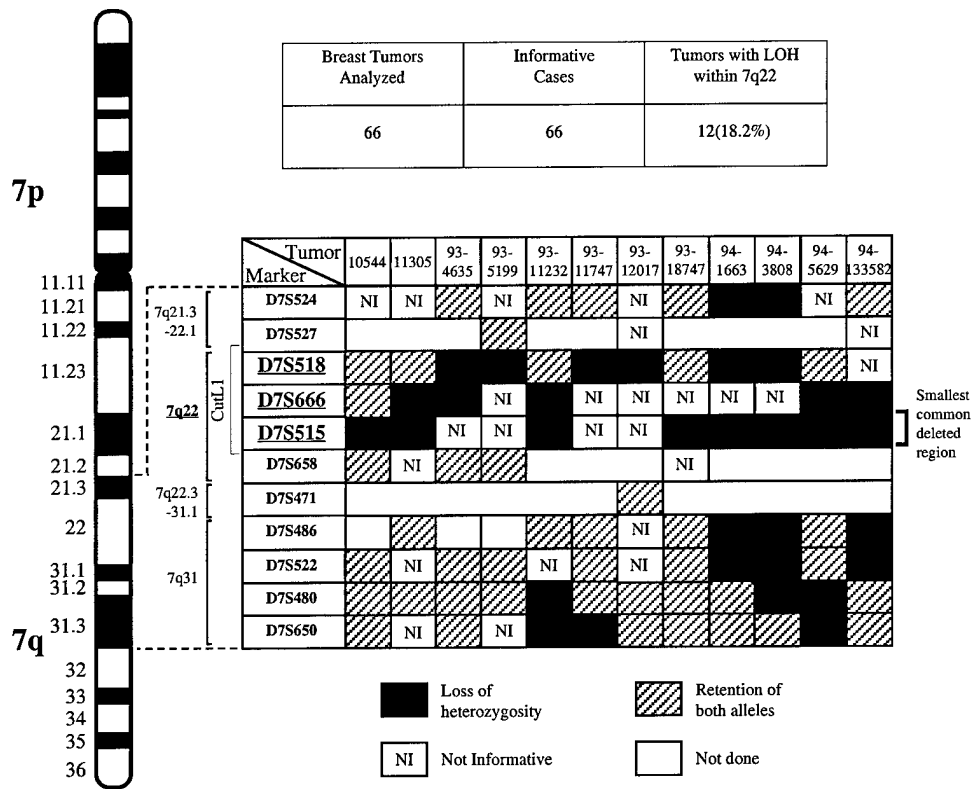


Fig. 4 Mapping of LOH Within 7q in Breast Tumors from the Manitoba Breast Tumor Bank
(Left) Representation of human chromosome 7 including band assignments. (Right) Names of polymorphic markers used are given, along with chromosome band assignments and the position of the CUTL1 gene. Twelve columns representing twelve patients with a loss of heterozygosity on chromosome 7q are shown. Explanation of the symbols used is given. The smallest common deletion in these seven patients is indicated by the vertical line to the right. All 54 tumors not shown in this figure were informative for at least one marker within the critical region but failed to show LOH.

Association Between LOH at 7q22 and Clinical Parameters

To determine whether loss of genetic material at 7q22 or 7q31 may be associated with pathological features of breast tumors, we analyzed available clinical data including the tumor type, grade and size, estrogen and progesterone receptor (ER and PR) status, occurrence of lymph node metastase and age of onset. We found no correlation between LOH at 7q22 and tumor type or grade, ER or PR expression, nodal status, or age of onset. However, there was a significant association (p value = 0.0082) between LOH at 7q22 and tumor size (Table 4). The average sizes of breast tumors with or without LOH at 7q22 were 4.99 and 2.93 cm respectively. Moreover, among breast tumors with LOH at 7q22, those without deletion at 7q31 had a larger size (6.5 Vs 3.5 cm). We are currently assessing whether a correlation can be found between LOH at 7q31 and any clinical feature.

Table 4 Relationship Between Allelic Loss for Loci at 7q22 and Clinicopathological Characteristics of the 66 Breast Tumors

Clinical-Pathological Characteristics		LOH 7q22		
		+	-	P value
Type	Ductal	10	46	ns
	Lobular	1	4	
	Other	sa	co,pa,is,is	
Grade	well	0	10	ns
	mod	6	22	
	poor	5	20	
	not graded	1	2	
ER	positive	7	36	ns
	negative	4	17	
PR	positive	5	24	ns
	negative	6	29	
	not assayed	1	4	
LN Status	positive	4	17	ns
	negative	8	32	
	unknown	-	4	
Size	mean (cms)	4.99	2.93	0.0082
Age	mean (yrs)	53	59	ns

Grade = Nottingham grade
 Sa = sarcoma, co = colloid carcinoma, pa = papillary carcinoma, is = predominant ductal carcinoma in-situ with small ductal invasive foci, ns = not significant, ER = estrogen receptor, PR = progesterone receptor, LN = lymph node.

The results of our LOH study form the basis of a manuscript that was submitted to the journal *Oncogene*. This manuscript is currently under revision and the revised version should be submitted in the next 6 weeks.

CONCLUSIONS

LOH within the 7q31 region has previously reported in human breast carcinomas, and in several cases the deletions encompassed the adjacent region, 7q22 (33, 34, 36-39). The results of the present study establish that in some breast tumors the loss of genetic material on the long arm of chromosome 7 can be limited to the 7q22 region. Thus, regarding chromosomal deletions on 7q, collectively the available data define three classes of breast tumors on the basis of whether the LOH region encompasses markers in only 7q22, only 7q31 or both 7q22 and 7q31 (33, 34, 36-39). These results suggest that 7q contains at least two tumor suppressor genes that can be inactivated in breast cancers, one at 7q22 and one at 7q31.

The smallest commonly deleted region on 7q22 includes polymorphic markers that are all located within the CUTL1 gene. Therefore, the tumor suppressor gene at 7q22 either is CUTL1 or is located close to it. Interestingly, in breast tumors that arise in MMTV-PyV LT transgenic mice, the murine Cut protein was found to form a complex together with the PyV LT antigen (30). Moreover, in cotransfection studies, the CUTL1 gene product was also coimmunoprecipitated together with the SV40 Large T antigen (SV40 LT) (Martin et al., manuscript in preparation). At present the effect of Large T oncoproteins on Cut function is not clear. However, these viral oncoproteins have previously been found to inactivate the function of the P53 and pRB tumor suppressor proteins. Thus, interactions with these viral oncoproteins suggest that Cut proteins may play an important role in the control of cellular proliferation. This hypothesis received further support from the finding that Cut DNA binding activity is regulated in a cell-cycle dependent manner (Coqueret et al., manuscript submitted). Whereas Cut was expressed in all phases of the cell cycle, Cut DNA binding activity was the highest at the end of G1 and during S phase.

In conclusion, two sets of data, LOH mapping analysis and protein-protein interaction studies, strongly point towards the CUTL1 gene as a candidate tumor suppressor gene. It should be stressed, however, that the CUTL1 gene appears to cover a very large distance, over 200 Kbp, and that several introns are more than 10 Kbp (Soucie et al., manuscript in preparation). It is thus possible that another transcription unit exists within the boundaries of the CUTL1 gene. In accordance with the recently proposed rules for the definition of a tumor suppressor gene, a firm statement about the identity of the tumor suppressor gene at 7q22 will have to await the demonstration either that the remaining allele is mutated or that the function of the CUTL1 gene product is inactivated or altered in some breast tumors (40). At the minimum, the fact that a polymorphic marker within CUTL1 is consistently deleted in breast tumors with LOH of 7q22 will provide a useful start point for positional cloning approaches to identify the critical tumor suppressor gene at 7q22.

There was no correlation between LOH of 7q22 and tumor grade, suggesting that the loss of genetic material at 7q22 is probably an early event in tumor development and, most likely, is not associated with tumor progression. On the other hand, LOH of 7q22 was associated with increased tumor size, a finding compatible with the notion that the tumor suppressor gene at 7q22 functions to restrict cellular proliferation. One of our goals when we initiated this study was to determine whether LOH of CUTL1 could be used as a prognostic marker for breast tumors. We did not observe a correlation between LOH of 7q22 and tumor grade or lymph node status. Therefore, we must conclude that LOH of CUTL1 could not be used as a prognostic marker for breast tumors.

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